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13. ABSTRACT *(Maximum 200 Words)*

In the Lewis rat, inhalation of silica (SL) in realistic doses for 6 wk exacerbated the Mycobacterium-induced autoimmune adjuvant disease and impaired the humoral as well as cellular immune responses. In addition to the lung, significant SL deposition was observed in the spleen and the brain. In the lung, SL exposure resulted in granulomatous and fibrogenic changes, and decreased airway hyperresponsiveness. In addition to the increased number and changes in the surface phenotype, alveolar macrophages exhibited strong anti-apoptotic responses. The bronchoalveolar lavage fluids had moderate (metalloproteinase-9) to highly significant (metalloproteinase-2) increases in extracellular matrix digesting enzyme activities. We also observed that the anti-nerve gas agent, pyridostigmine bromide (PB), affected the immune system only when it was administered directly into the brain, indicating that PB and other cholinergic agents are unlikely to affect the immune response unless they cross the blood-brain-barrier. Future experiments will be directed to understand the mechanism of anti-apoptotic responses in alveolar macrophages. This response may be crucial for the development of SL-induced lung pathology. In addition, experiments are planned to ascertain the mechanism and the effects of SL sequestration in the brain.

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I. INTRODUCTION

During Operation Desert Storm, American soldiers were exposed to respirable Saudi Arabian sand dust containing a high percentage of silica (SL) and organic matter. On their return from the Persian Gulf, some of the soldiers developed a set of miscellaneous symptoms that were termed as the Gulf War Syndrome (GWS). Various hypotheses, still awaiting proof, have been proposed to explain the GWS. We proposed that in susceptible individuals, inhalation of SL (an immune adjuvant) might produce the GWS-like symptomatology through over-activation of the immune system, leading to autoimmune adjuvant disease (AAD). These effects might be exacerbated in the presence of organic matter and/or the anti-nerve gas agent, pyridostigmine bromide (PB). This hypothesis that SL causes AAD was tested in Lewis rats exposed to moderate doses of SL for 6 wk. Our experiments with SL-exposed animals indicate that the biological effects of SL are dependent on the time following the 6-wk SL exposure. Therefore, the 6-wk point was taken as 0 time and observations were made at various times after 0 time. Results from these experiments suggested a novel immunological mechanism for the SL-induced lung pathology. Details of the experimental results are presented in the next section.

II. BODY OF THE REPORT

A. Silica Exposure

SL (SiO_2 or SL) was obtained as a gift from U.S. Silica (Berkley Springs, WV) and ground to an average size of 1.5 μ. Lewis rats were exposed to fresh air (CON) or airborne SL (SL) at 5 mg/m^3 for 6 h/day, 5 days/wk for 6 wk. This concentration of SL is 15 times the human Threshold Limit Value for SL. However, the pulmonary deposition of SL in the rat is much lower than in humans exposed to the same level of SL. In fact, as given in Table 1, the lung SL burden of rats after 6 wk of SL exposure (0 time) was much lower (465 ± ng/mg tissue) than some patients with silicosis (2240 ± 410 ng/mg tissue) (1). Therefore, in terms of human exposure, this dose of SL for exposing rats was very realistic. Significant amounts of SL were also detected in the spleens and brains of exposed animals. Interestingly, at 12 wk after the exposure, polarized microscopy found an occasional SL particle in the lung, but SL was undetectable by mass spectrometer analysis (Table 1). **Thus, animals inhaling realistic doses of 1.5 μ SL particles have the ability to clear SL from their tissues.** This conclusion would have been hard to infer from most published animal studies due to the use of very high concentrations of SL in most studies. Human lungs may also be able to clear low doses of SL (2).

Table 1
SL Burden (ng /mg tissue)

Time After Exposure	Lung	Spleen	Brain	Liver
0 Time				
CON[a]	97 ± 26	79 ± 13	36 ± 2	49 ± 20
SL	413 ± 59	708 ± 154	186 ± 48	ND
12 Weeks				
SL	111 ± 8	51 ± 3	38 ± 3	50 ± 13

[a]CON = control animals; SL = silica-exposed; ND = not done

Tissues were sent for SL determination to the Carlsbad Environmental Monitoring & Research Center (New Mexico State University). After digestion of tissues, SL concentrations were determined by inductively coupled plasma-mass spectrometer following the EPA Method 200.8.

B. Histopathological Analysis of SL-Exposed Lungs

Occupational exposure to SL by inhalation may lead to pneumoconiosis and pulmonary fibrosis, and it is generally believed that the SL-induced lung lesions are progressive. However, there are reports that the lung pathology in some silicosis patients may be partially reversible. Histology of the lungs from rats at various times after SL exposure suggests that SL induced granulomas in the lung several weeks after the exposure; however, the size of the granulomas decreased after SL was cleared from the tissues. This might primarily reflect the depletion of leukocytes within the granulomas. In fact, 37 wk after SL exposure, granulomas were much smaller and mainly composed of epithelial cells. It is entirely possible that these granulomas would eventually disappear. **Thus, the granulomatous changes in SL-treated animals are not necessarily irreversible, and the probability to regress may depend upon the ability of the host to clear SL from the lung. A complete description of histopathology findings is provided in Appendix 1.**

C. SL Exacerbates Mycobacterium-Induced AAD

CON and SL-exposed rats were injected in the tail vein with vehicle (mineral oil) or mineral oil mixed with the extracts of heat-treated *Mycobacterium smegmatis* (3.5 mg/animal). Two weeks after SL inhalation, histopathological examination of the leg joints from SL-treated or SL + mineral oil-treated (Fig. 1, left panel) animals did not show any signs of inflammation (i.e., synovitis, tendonitis, myositis, fascitis, periostitis, and cellulitis). On the other hand, rats treated with Mycobacterial extracts (Fig. 1, center) had weak to moderate inflammation (synovitis and fascitis), but all SL-treated animals that were injected with Mycobacterial extracts, showed moderate to severe inflammation (Fig. 1, right). **These results indicate that SL acts as an adjuvant and exacerbates Mycobacterium-induced AAD.**

Fig. 1 *SL aggravates joint inflammation caused by mycobacterial extracts. Results from a representative animal from each group are provided: CON animal injected with mineral oil (left), CON animal injected with Mycobacterium extract (center), and a SL-exposed animal injected with Mycobacterial extract (right).*

D. SL Impairs Adaptive Immune Responses

AAD is an immunopathologic condition. Because SL increased the Mycobacterium-induced AAD, we examined the humoral (antibody-forming cell [AFC] response to sheep red blood cells [SRBC]) and cell-mediated (Con A-induced T cell proliferation) immune responses in SL-treated animals at 0-time and 3 wk after SL exposure. As seen in Fig. 2A, SL significantly increased the ability of splenocytes to produce AFC against the T cell-dependent antigen, SRBC. Similarly, SL stimulated the Con A-induced mitogenesis of T cells (Fig. 3A). However, the immunological changes induced by SL were reversed after SL was cleared from the tissues (Figs. 2B and 3B). **These results strongly suggest that the adjuvant effects of SL are not restricted to adjuvant arthritis, but that SL is a broad-spectral immunoadjuvant. This could partly explain the exacerbation of autoimmune conditions among some participants of the Gulf War. Interestingly, however, the immunostimulation was detected only during the presence of SL in the tissues and reverted to a normal response after it cleared.**

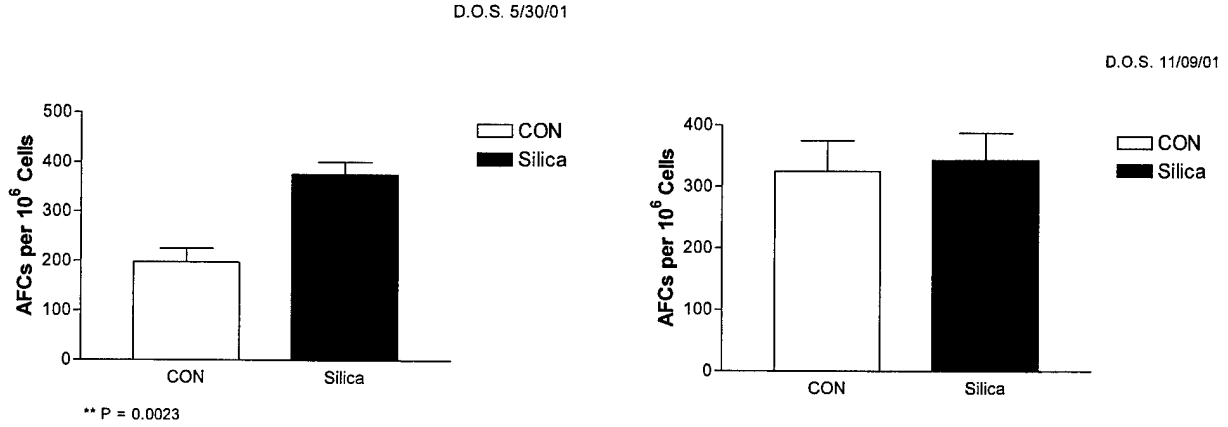


Fig. 2 *SL stimulates the AFC response to SRBC.*

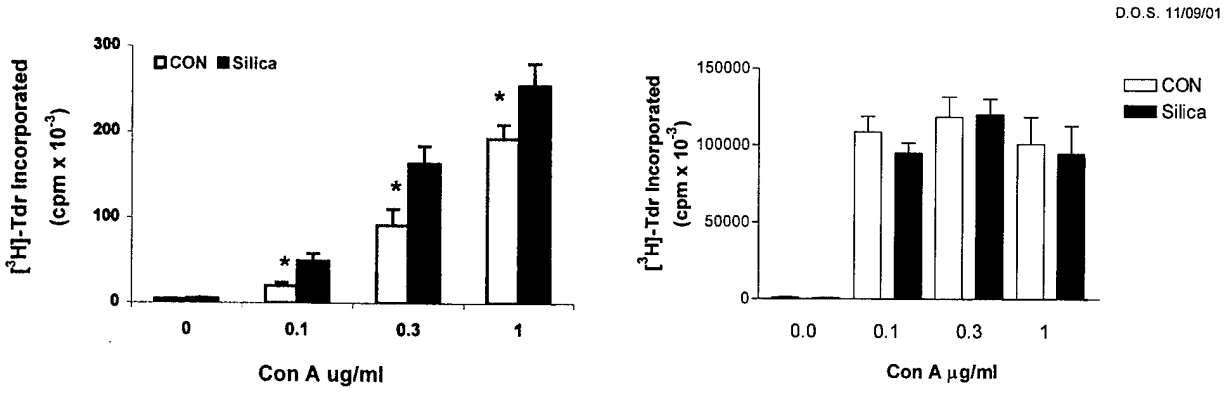


Fig. 3 *SL increases the Con A-induced T cell mitogenesis.*

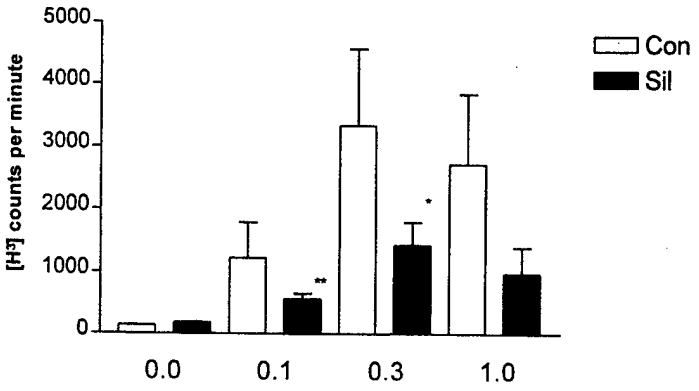


Fig. 4 *SL decreases the Con-A induced T-cell mitogenesis in PBMC.*

A surprising finding in SL-treated animals at 0-time was a significant decrease in proliferation of peripheral blood mononuclear cells (PBMC) and a significant increase in proliferation of spleen cells in response to the T cell mitogen, Con A (Fig. 4 and Fig. 3A). It is possible that SL accumulated within lymphoid tissues and activated the immune system, while the absence of significant levels of SL in the blood, did not allow immunostimulation. Indeed, significant quantities of SL were present in the spleen, which showed the immunoadjuvant effects of SL. It was possible that differences in the immunological responses between the spleen and PBMC resulted from SL-induced changes in blood glucocorticoid levels, leading to immunosuppression. However, the plasma corticosterone levels were not different between SL-treated and CON animals (not shown). Moreover, as seen with spleen cells, the effects of SL on the PBMC Con A response was lost after SL was cleared from the tissues (not shown).

E. Changes in the Composition of Bronchoalveolar lavage (BAL) After SL Exposure

Inhalation of SL can result in lung inflammation that may progress to fibrosis. The mechanism of pulmonary silicosis is largely unknown, however, chronic inflammation is associated with mononuclear cell infiltrates and alveolar macrophages may play an important role in this process. We examined the milieu of BAL fluids after 0-time in SL-exposed and CON animals, and these findings are summarized below.

1. <u>SL Stimulates Infiltration and Activation of Macrophages in the BAL</u>

As pointed out earlier, other than a dramatic increase in the number of BAL cells, at 0-time SL-treated animals did not show significant histopathological change in the lung tissue. By differential staining, cells were >98% macrophages and noticeably larger than BAL cells from CON animals (Fig. 5), suggesting that SL activates alveolar macrophages. Protein content and the lactate dehydrogenase (LDH) activity of BAL cell-free supernatants from SL-treated animals were not significantly different from CON animals (Table 2). **Thus, at 0-time SL did not cause overt pulmonary damage.**

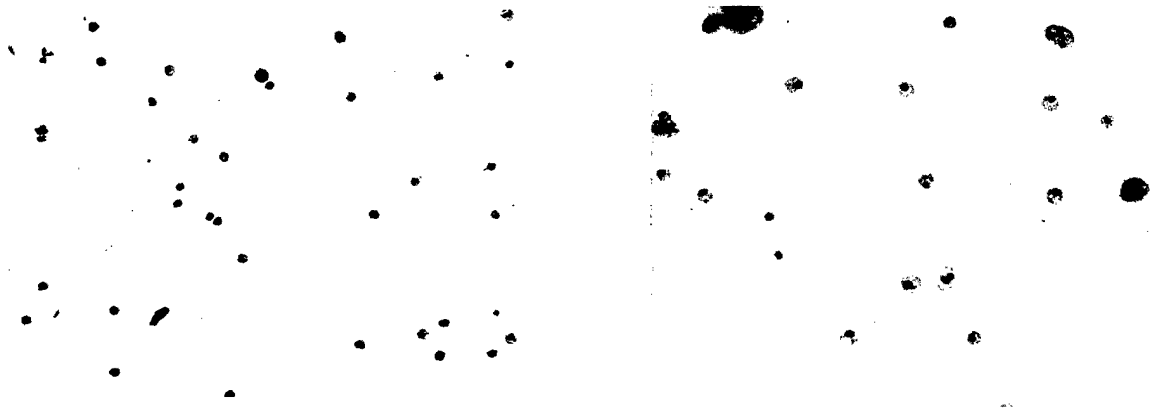


Fig. 5 *SL stimulates infiltration of activated macrophages into the BAL.*

Table 2
LDH and Total Protein

Time After Exposure	LDH	Total Protein
0 Time		
CON	38.0 ± 4.0 U/L	2.0 ± 0.3 mg/DL
Silica	44.0 ± 3.0 U/L	2.3 ± 0.2 mg/DL
27 Weeks		
CON	23.4 ± 2.0 U/L	2.4 ± 0.4 mg/DL
Silica	100.6 ± 24.0 U/L*	6.4 ± 0.7 mg/DL**

2. SL Stimulates Infiltration of Neutrophils and Lymphocytes

By differential staining, BAL cells from CON lungs were essentially all (95%) macrophages. By double labeling, these cells were analyzed for the expression of CD11a (LFA-1) and CD11b (Mac-1) surface markers by flow cytometry using rat-specific PE-conjugated anti-CD11a and FITC-conjugated anti-CD11b monoclonal antibodies. Normally, LFA-1 is present primarily on lymphocytes and Mac-1 on neutrophils and peritoneal macrophages. Interestingly, however, unlike peritoneal macrophages, most of the lung macrophages were double positives with <10% CD11b and <5% CD11a single positives (Fig. 6). **Thus, the surface expression of BAL macrophages ($CD11a^+/CD11b^+$) is distinct from the peritoneal macrophages ($CD11a^-/CD11b^+$).** Following, SL treatment, differential staining indicated the infiltration of neutrophils and lymphocytes into the BAL, and flow cytometric analysis showed a significant increase in the population of single positive cells (i.e., $CD11a^+/CD11b^-$ and $CD11a^-/CD11b^+$).

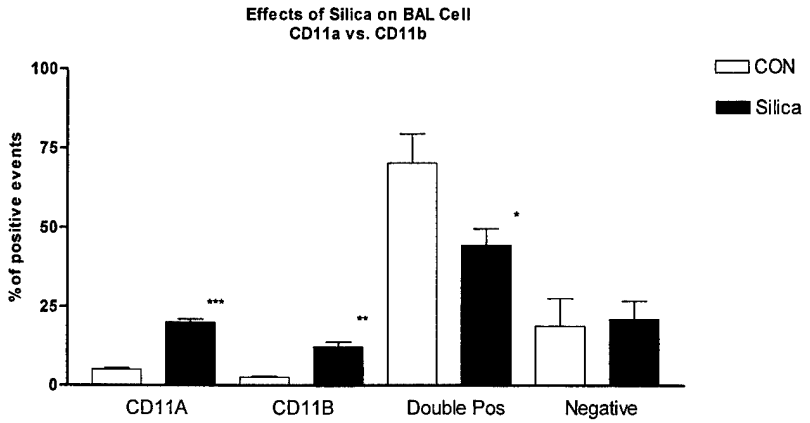


Fig. 6 *Cell surface phenotype of BAL cells before and after SL exposure.*

3. SL Induces Anti-apoptotic Phenotype in BAL Cells

SL causes lung fibrosis and increases the incidence of lung cancer (3), however, the molecular events in these responses have not been defined. Alveolar macrophages may play an important role in this process (4). While *in vivo* and *in vitro* experiments have attempted to define parameters associated with these processes, the doses of SL used were generally unrealistic. Our experiments, for the first time, suggest that chronic inhalation of small amounts SL may affect the proliferation and life span of alveolar macrophages, and these long-living macrophages may continue to affect the inflammatory responses in the lung. The following experiments suggest that BAL cells, representing >80% macrophages, display a strong anti-apoptotic phenotype.

a. SL Increases the Expression of Anti-apoptotic Bcl-2

Bcl-2 is an anti-apoptotic molecule that inhibits the release of cytochrome c from the mitochondria into the cytoplasm. This event is important in the activation of caspases that are effector molecules of apoptosis. Fig. 7 shows that **BAL cells from SL-treated animals have very high concentration of the anti-apoptotic protein, Bcl-2.**

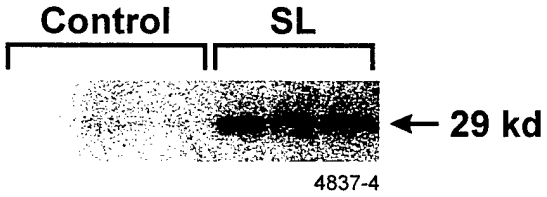


Fig. 7 *SL increases the concentration of the anti-apoptotic molecule BCL-2.*

b. SL Inhibits the Conversion of Pro- to the Active Form of Caspase-3

All caspases are made as zymogens and must be activated to be effective. Caspase-3 is a critical effector molecule in apoptosis. As seen in Fig. 8 the zymogen form of caspase-3 (procaspase-3) is significantly higher in BAL cells from SL-treated than from sham CON animals. Moreover, Bal cells from SL-treated animals have almost undetectable

levels of the active caspase. **These results suggest that a key effector components of apoptosis (i.e., caspase 3) is present almost entirely in the inactive zymogen form.**

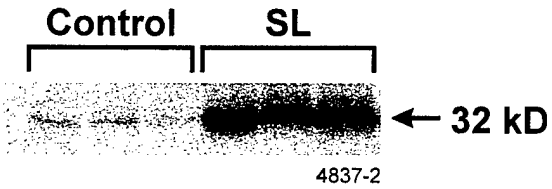


Fig. 8 *SL increases the concentration of the molecule procaspase-3 in BAL cells.*

c. SL Inhibits the Stress-Activated Protein Kinase (SAPK)

Many agents that cause cell cycle arrest (e.g., ionizing radiations, p53) induce apoptosis that is inhibited by Bcl-2 and Bcl-X_L (5, 6). SAPKs, also termed as c-Jun amino terminal kinases (JNKs) and P32 MAP kinase are also activated by a diverse set of proapoptotic stimuli such as DNA damage, heat shock, IL-1, TNF-α, and Fas (7–9). Activation (phosphorylation) of SAPK translocates it to mitochondria, where it phosphorylates anti-apoptotic members of Bcl-2 family (e.g., Bcl-X_L), promoting release of cytochrome c, activation of caspases, and apoptosis. Because SL increased the intracellular concentration of Bcl-2 and procaspase-3, we determined whether it resulted from changes in SAPK expression/activity. Fig. 9 shows that SAPK is almost absent in SL-treated BAL cells, **suggesting that SL might control apoptosis by regulating the expression of SAPKs**.

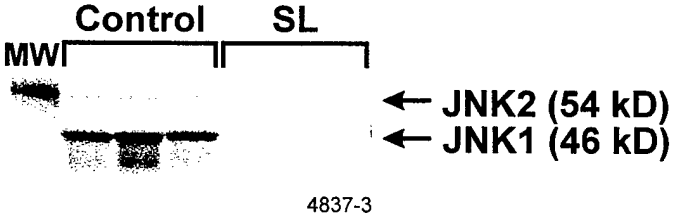


Fig. 9 *SL significantly decreases the concentration of SAPKs JNK1 and JNK2 in BAL cells.*

4. SL Increases Metalloproteinase MMP-2, MMP-9, and Alkaline Phosphatase Activities in BAL Fluids

MMPs and alkaline phosphatase have been linked to fibrosis (10). Our studies indicate that SL increases MMP-2, MMP-9, and alkaline phosphatase activities in BAL fluids (Fig. 10). While there was only a modest increase in MMP-9, SL caused a dramatic increase in the MMP-2 activity (Fig. 11). Interestingly, MMP-9 but not MMP-2 activity, is increased in an animal model of cigarette smoke-induced emphysema (10, 11). **Therefore, it is possible that MMP-2 might be a biological marker for SL-induced fibrotic changes in the lung.** Moreover, MMP-2 cleaves FasL that is essential for the Fas-FasL activation of apoptosis. Therefore, MMP-2 could further inhibit the apoptotic process of BAL cells. Moreover, MMP-2 and MMP-9 have been implicated in tumor metastasis (12). **SL inhalation is a risk factor in lung cancer (3), and increased MMP activities might contribute to lung tumorigenesis.**

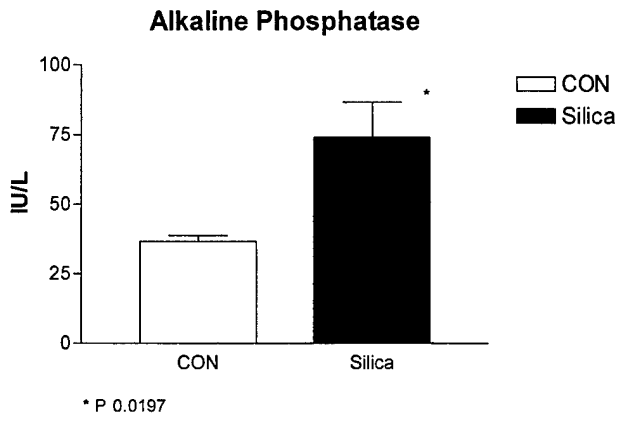


Fig. 10 *SL increases the level of alkaline phosphatase activity in BALF.*

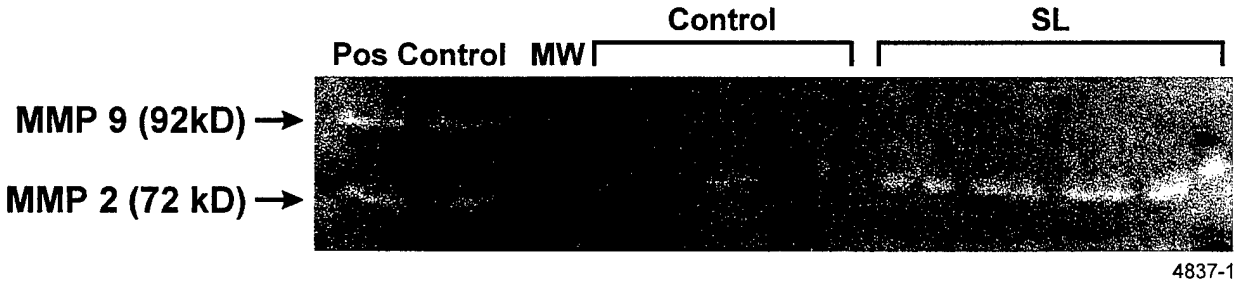


Fig. 11 *SL dramatically increases the level of MMP-2 in BALF.*

F. SL Decreases Airway Hyperresponsiveness (AHR)

Because SL produces granulomatous changes in the lung, we expected that it might increase AHR due to increased resistance to airflow. Surprisingly, AHR to methacholine was significantly reduced in SL-treated animals (Fig. 12). This suggested the granulomatous changes in the lung did not block the airways. In fact, SL-induced granulomas are peripheral in the lung and do not block the airways. However, the reason for decreased AHR is not clear at this time. It is possible that granulomas affected the lung elasticity, thereby reducing the ability of the lung to hold air (i.e., decreasing the pause or PenH value during exhalation). Alternatively, alveolar macrophages from SL-treated animal produced large amounts of nitric oxide, a powerful bronchodilator that relaxes the lung musculature and decreases resistance to airflow. Nonetheless, we expect that the SL-treated animals would have reduced pulmonary function.

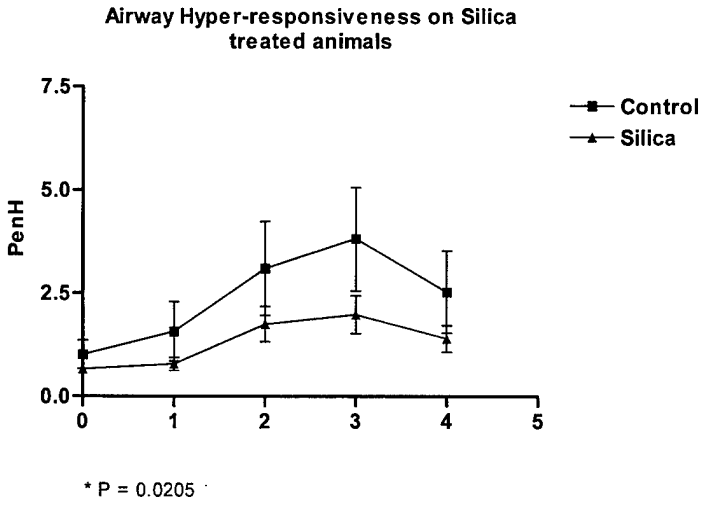
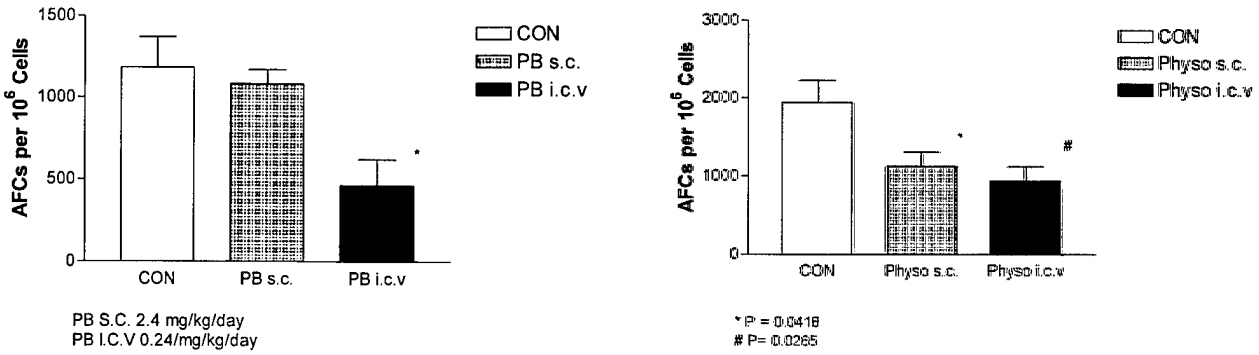


Fig. 12 *AHR response is significantly decreased in SL-treated animals when challenged with varying concentrations of methacholine.*

G. Immunomodulatory Effects of PB are Mediated Through the Brain

Pyridostigmine bromide (PB), a reversible anti-cholinesterase drug, was used as a prophylactic against possible nerve gas exposure during the Gulf War. Cholinergic agents may affect the communication between the brain and the immune system (13), and we have observed that the cholinergic agent, sarin (nerve gas), impairs the neuroimmune communication (14). Our experiments with PB suggested that, when given peripherally via subcutaneously implanted miniosmotic pumps, it has no detectable adverse effects on immune responses (Fig. 13). However, intracerebroventricular administration of PB resulted in the suppression of the AFC response. Using other cholinergic agents including physostigmine (Fig. 14), sarin, and malathion (data not shown), **we have concluded that cholinergic agents must reach the brain to affect the immune system. Therefore, when given peripherally, cholinergic agents that do not cross the blood-brain-barrier (e.g., PB) do not significantly affect the immune system.**



Figs. 13 and 14 *PB delivered s.c. via miniosmotic pump has no effect on AFC response. However, when delivered i.c.v. causes moderate immuno-suppression. This immuno-suppression is also seen in physostigmine (s.c. and i.c.v.).*

III. KEY RESEARCH ACCOMPLISHMENTS

- Inhaled SL accumulates in tissues including the lung, the spleen, and the brain. In the lung, exposure to realistic doses of SL produce granulomas. However, over a period of time, SL was cleared from the tissues, and this clearance might account for the decreases in the size of granulomas. To our knowledge, we are the first to make this observation.

- SL has been previously shown to be an immunoadjuvant. Those studies were carried out by inhalation or intratracheal deposition of extremely high doses of SL. We confirmed the immunostimulatory effects of SL on spleen cells using multiple immunological endpoints (i.e., SL enhances AAD, and both humoral and cell-mediated immunity). However, surprisingly, the Con A response of PBMC from SL-treated animals was significantly lower than CON animals (a new finding). While the mechanism of this duality is not known, it did not appear to result from significant changes in surface phenotype of PBMC.

- Intratracheal instillation of a bolus dose of SL (20 mg/mouse ≈800 mg/kg body wt) was shown to cause increased production of TNF-α in the BAL and Fas-related apoptosis in the lung (Borges et al., 2001). In our experiments, SL exposure produced a SL burden of only ~400 μg/g of lung tissue. While we have not yet tested the Fas-FasL pathway, our results clearly indicate that these doses of SL strongly suppressed BAL cell apoptosis through the SAPK pathway. This could increase the half-life and numbers of mononuclear cells in the lung, exacerbating granuloma formation. Moreover, our preliminary experiments suggest that BAL fluids from SL-treated animal have significantly lower TNF-α levels than in CON animals. If confirmed, the observation would imply that exposure to SL suppresses many modulators of apoptosis in alveolar macrophages, including the production of TNF-α. It is possible, however, that while SL stimulates the expression of anti-apoptotic proteins in lung leukocytes, it triggers pro-apoptotic pathway(s) in other lung cells (e.g., epithelial cells).

- Metalloproteinases such as MMP-2 and MMP-9 dissolve extracellular matrix proteins, causing lung injury and promoting tumor metastasis. The evidence that SL stimulates the production of MMP-2, MMP-9, and alkaline phosphatase suggests that SL may promote lung injury through its effects on structural proteins. A dramatic increase in the activity of MMP-2 might affect cell survival through breakdown of FasL. Because cytotoxic T cells and natural killer cells kill bacteria/virus-infected cells and tumor cells through FasL-Fas interaction, SL might compromise the ability of the lungs to dispose of pathogens and tumor cells. The latter effect, together with decreased apoptosis, might explain the increased risk of lung tumors among silicosis patients.

- We have clearly shown that subcutaneous exposure of rats to PB for 3 to 4 wk, at doses comparable to those consumed by the Gulf War Veterans, did not affect either the humoral (AFC) or the cell-mediated (Con A) immune responses. However, cholinergic agents might have to cross the blood-brain-barrier to affect the immune system. Moreover, unlike most xenobiotics, cholinergic agents strongly suppressed glucocorticoid synthesis. Therefore, decreased blood glucocorticoid levels might be a biological marker for cholinergic exposure.

IV. REPORTABLE OUTCOMES

We have many interesting observations that should enable us to eventually write several papers. While some observations may need additional experiments to support the inferences, the following three manuscripts are expected be submitted for publication within the next 2 months:

Raymond J. Langley, Shashi P. Singh, Roma Kalra, Fletcher Hahn, Seddigheh Razani-Boroujerdi, Juan C. Philippides, and Mohan L. Sopori. Effects of SL Inhalation on the Immune System. I. SL Stimulates the Splenic but Inhibits the PBMC Immune Responses.

Raymond J. Langley, Neerad Mishra, Satya Saxena, and Mohan L. Sopori. Effects of SL Inhalation on the Immune System. II. SL Activates and Promotes Anti-apoptotic Phenotype in Bronchoalveolar Mononuclear Cells.

Raymond J. Langley, Roma Kalra, Shashi P. Singh, Seddigheh Razani-Boroujerdi, Juan C. Philippides, and Mohan L. Sopori. The Central but not Peripheral Cholinergic Activity Suppresses the Immune System.

V. CONCLUSIONS

Chronic exposure to small doses of SL had powerful effects on the innate and adaptive immune responses, which could explain its immunoadjuvant as well as cancer-promoting properties. In addition, SL was also deposited in the brain and, as it did with alveolar macrophages, it might activate brain microglia to produce inflammation in the brain. Such events might compromise the blood-brain-barrier, allowing prophylactic drugs such as PB to enter the central nervous system and modulate neuroimmune communication. Moreover, based on our studies with PB, sarin, and other cholinergic agents, it is possible that dramatic drops in blood glucocorticoid levels will become a biomarker for cholinergic toxicity.

At this time, we know very little about the mechanism by which SL alters the immune system. It is possible, however, that ingestion of SL by alveolar macrophages and perhaps microglia results in the production of cytokines that affect the inflammatory responses in the lung and the central nervous system. In that context, several reports have observed encephalopathy or other neurological disorders in some silicosis. Studies from other laboratories indicate that SL is not equally toxic in all humans or inbred mouse strains. Therefore, genetic factors may govern the susceptibility to SL-induced immunological changes. Evidently, the immune system of Lewis rats, which were used in this study, is susceptible to relatively low concentrations of SL. For the arguments presented in the original grant application, we believe that F344 rats might be less sensitive to immunotoxicity of SL. If that proves to be the case, it could allow identification of the susceptibility factors using molecular approaches such as microsatellite, gene array, and proteomics analysis.

VI. FUTURE STUDIES

The original application was approved for a 3-year funding period; however, we received only a fraction of the requested funds (primarily for demonstrating the feasibility of the proposed study). We believe our results in the past year show this research to be highly viable with implication for both civilian and military health. These studies could also help in understanding the fibrogenic responses in other lung diseases (e.g., tuberculosis, chronic

obstructive pulmonary disease). We will continue to use the same SL exposure protocol (i.e., the 6-wk exposure at 5 mg/m^3), and seek funds for the next 3 years to explore the following:

- Determine the precise kinetics of the anti-apoptotic process in BAL cells and correlate it with changes in the expression of various proinflammatory/anti-inflammatory and pro-fibrogenic cytokines and chemokines.

- Determine whether the anti-apoptotic processes in BAL cells induce proapoptotic response in other lung cells. (We will use immunohistochemistry to identify and score apoptotic cells in the lung and test BAL cells in cell and organ cultures of the lung tissue).

- Identify the signaling pathways that impart anti-apoptotic phenotype to BAL cells. We will examine both the intrinsic and extrinsic (Fas-FasL) pathways.

- Determine whether the decrease in AHR is associated with the loss of lung function (i.e., changes in FEV-1).

- In addition to the lung, SL is also sequestered in the brain and spleen; we will evaluate these tissues by histopathology, immunohistochemistry, and RT-PCR/RPA for inflammation, gliosis (brain), type of cells containing SL, and the expression of various cytokines. Because spleen cells are much easier to obtain, they may help in identifying the mechanism(s) of silicosis.

- There is a possibility that SL affects the integrity of the BBB; we will determine whether exposure to SL facilitates the entrance of PB and influenza virus into the brain.

We estimate 3 batches of SL-exposed animals would be required (one batch every 6–8 months) to perform the proposed experiments (see VIII. COST ANALYSIS).

VII. REFERENCES

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VIII. COST ANALYSIS

Cost Estimate for Army
Silica Exposure

	Year 1		Year 2		TOTAL	
Personnel	Effort	$	Effort	$	Effort	$
M. Sopori	0.20		0.20		0.20	
R. Langley	1.00		1.00		1.00	
J. Carlos	0.50		0.50		0.50	
Subtotal	**1.70**	**71,363**	**1.70**	**74,932**	**1.70**	**146,295**
Total Labor		**71,363**		**74,932**		**146,295**
Leave Benefits @ 14%		9,991		10,490		20,482
Fringe Benefits @ 24%		19,525		20,501		40,026
Total Labor Cost		**100,879**		**105,923**		**206,803**
Travel		3,000		3,000		6,000
Exposure Charge		32,270		35,380		67,650
Supplies/Services						
Animals		14,400		14,400		28,800
Per Diem		21,600		21,600		43,200
Kits		7,605		7,605		15,210
Antibodies		13,545		13,545		27,090
Flow Cytometer Charges		2,400		2,400		4,800
Plastic and Glassware		3,000		3,000		6,000
Cell Culture Supplies		3,000		3,000		6,000
Biochemicals and Radio Chemicals		3,000		3,000		6,000
Histopathology		10,350		10,350		20,700
Necropsy		3,000		3,000		6,000
Subtotal		**81,900**		**81,900**		**163,800**
Other Expenses						
Computer service		5,078		5,332		10,410
Subtotal		**5,078**		**5,332**		**10,410**
Total Direct Cost		223,127		231,535		454,663
Indirect Cost		223,127		231,535		454,663
Total Price		**446,254**		**463,070**		**909,326**

Price estimate prepared 03/12/2002 by F. Olivas

IX. APPENDIX

HISTOPATHOLOGY SUMMARY

F. F. Hahn – February 26, 2002
RZ-217; Silica exposed 6 h/day, 5 days/wk, 6 wk to 5 mg/m^3
Sacrifices 4 days, 4 wk, 10 wk, 17 wk, 27 wk and 37 wk after end of 6-wk exposure

SUMMARY

A granulomatous pneumonia developed in the rats exposed to silica fiber, but only after 17 weeks following the six-week exposure period. The granulomas were characterized by focal accumulations of epithelioid macrophages surrounded by lymphocytes and occasional neutrophils. Using polarized light, silica fibers could be found in the macrophages through out the lung. The progression of microscopic changes leading to the granulomas appeared to be focal alveolar macrophages → lymphoid aggregates (around venules) → infiltration of AMs with neutrophils, lymphocytes → aggregation of epithelioid cells surrounded by macrophages, lymphocytes, PMN forming granulomas. The granulomas were concentrated in the periphery of the lung, in alveoli adjacent to the pleura. Epithelioid cells similar to those in the granulomas were found in the bronchial associated lymphoid tissues and bronchial lymph nodes before they were seen in the lung. Silica fibers were found in these epithelioid cells and were increased at longer times after the end of exposure. In the lung, however, the number of silica fibers decreased with time.

METHODS

Lung tissue sections, formalin fixed, paraffin embedded and stained with hematoxylin and eosin, were examined. Two or three cross sections were present for each rat. Bronchial lymph node sections, cut longitudinally, were examined where present. These lymph nodes were not routinely sampled at 10 and 37 wk after end of exposure. In addition, bronchial lymph nodes were not present in sections of a few rats sampled at other times.

The following criteria were used to grade the various histologic changes noted.

Microscopic Abnormalities in the Lung

Alveolar Septal Lymphoid Aggregates

1= 1–5 aggregates
2 = 6–10
3 = 11–20
4 = >20
Large aggregates may up grade by 1

Perivenous Lymphoid Infiltrates

1= 1–2 foci
2 = 3–5
3 = 6–9
4 = >9
Large foci may up grade by 1

Focal Aggregates of Alveolar Macrophages

1 = 1–5
2 = 6–15
3 = 16–30
4 = >30
Large foci may up grade by 1

Granulomas (focal accumulation of epithelioid cells, macrophages and lymphocytes)

1 = 1–5
2 = 6–15
3 = 16–30
4 = >30
Large granulomas may up grade by 1

Microscopic Abnormalities in Bronchial Associated Lymphoid Tissue

BALT Present

1 = 1–2 lymphoid foci
2 = 3–5
3 = 6–9
4 = >9
Enlarged foci may up grade by 1

Epithelioid Cells (in BALT or BLN)

1 = 1–5 aggregates
2 = 6–10
3 = 11–20
4 = >20

Silica Fibers (observed by polarized light in macrophages or epithelioid cells)

1 = Difficult to find
2 = Few

Microscopic Abnormalities in Bronchial Lymph Node

Hyperplasia

Relative size and lymphoid activity graded 1to 4

RESULTS

Controls –

At the end of the 6 wk exposure period, no microscopic abnormalities were noted in the lungs of the control rats. Only minimal microscopic abnormalities were noted in the lungs of a few rats sacrificed at 27 and 37 wk after the end of 6 wk of exposure to chamber air.

Exposed –

Table 1 summarizes the key histologic changes noted at the various sacrifice times after the end of the 6-wk exposure period. At the end of the exposure period (4 days), the only change in the lung is the presence of a minimal number of alveolar macrophage foci (Figure 1). The foci

contained a few to a dozen macrophages in alveoli usually at the periphery of the lung, adjacent to the pleura. Silica fibers could be found in some of these macrophages. No changes or fibers could be found in the brachial associated lymphoid tissue (BALT) or the bronchial lymph nodes (BLN).

At four weeks after end of exposure, lymphoid aggregates were present in the alveolar septa, usually associated with focal accumulations of alveolar macrophages (Figure 2). At 10 wk, neutrophils and lymphocytes were found in the alveoli with the macrophages and septal lymphoid aggregates. At this same time, epithelioid cells (large mononuclear cells with abundant eosinophilic cytoplasm) were present in the BALT. Using polarized light, a very few, short birefringent, needle-shaped fibers typical of silica could be found in the epithelioid cells.

Table 1
Microscopic Abnormalities in Rats Exposed to Silica Aerosols
for Six Weeks (Average Severity Grades)

	Lung					BALT		BLN	
Sacrifice Time[a]	Focal AM	Septal Lymphoid Agg.	Alveolar PMN/LYM	Granuloma	Fibers[b]	Epithelioid Cells	Fibers[c]	Epithelioid Cells	Fibers
4 days	0.4	0	0	0	1.2	0	0	0	0
4 wk	0.8	0.6	0	0	0.8	0	0	0	0.5[b]
10 wk	1.4	0.8	1.6	0	0.6	1.3	1.3	Not Sampled	
17 wk	3.0	1.2	2.8	0.4	1.0	0.8	0	4.0	2.0[c]
27 wk	2.5	1.0	2.5	2.0	1.0	1.2	1.0	4.0	1.8[c]
37 wk	1.5	1.0	1.0	2.0	0.2	0.7	0.2	Not Sampled	

[a]Time after end of 6-wk exposure.
[b]In alveolar macrophages.
[c]In epithelioid cells.

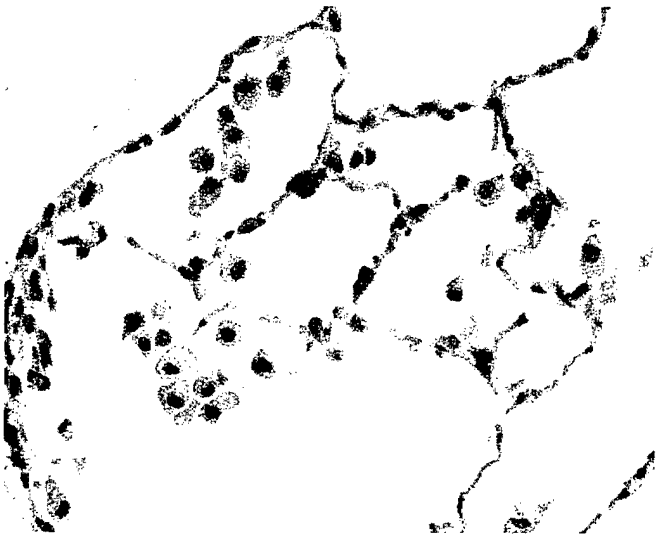

Figure 1. Focal accumulation of alveolar macrophage foci 4 days after end of exposure.

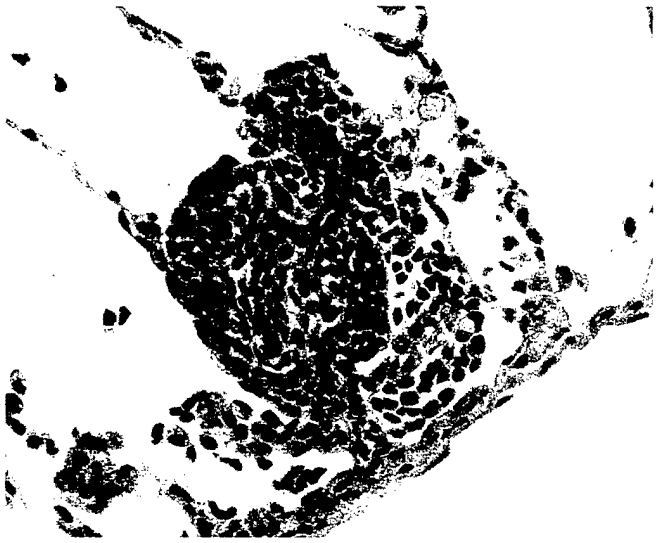

Figure 2. Aggregations of lymphocytes in alveolar septa and alveoli 4 wk after end of exposure.

At 17 wk after end of exposure, a few granulomas were present in the lung, concentrated in the pleura and adjacent alveoli. Some granulomas were randomly scattered through the parenchyma. However, there was not a predilection for the airways or terminal bronchioles. The granulomas were characterized by focal accumulations of epithelioid macrophages surrounded by lymphocytes and occasional neutrophils (Figure 3). In many, there were a few collagen fibers present. In some, the macrophages were necrotic and neutrophils are present in the granuloma. Focal accumulations of alveolar macrophages were scattered through the lung. In some of these foci, lymphocytes were present. Adjacent to some foci and granulomas, the alveolar epithelial cells are hypertrophic and hyperplasic, thickening the alveolar wall. Foci of epithelioid macrophages are present in the bronchial lymph nodes and, to a lesser degree, in the bronchial associated lymphoid tissue. In the lymph nodes, necrosis and fibrosis were associated with the epithelioid cell foci (Figure 4). Silica fibers could be found relatively easily in these cells in the node.

Figure 3. Granulomas adjacent to the pleura, characterized by epithelioid macrophages, lymphocytes and occasional neutrophils 17 wk after end of exposure.

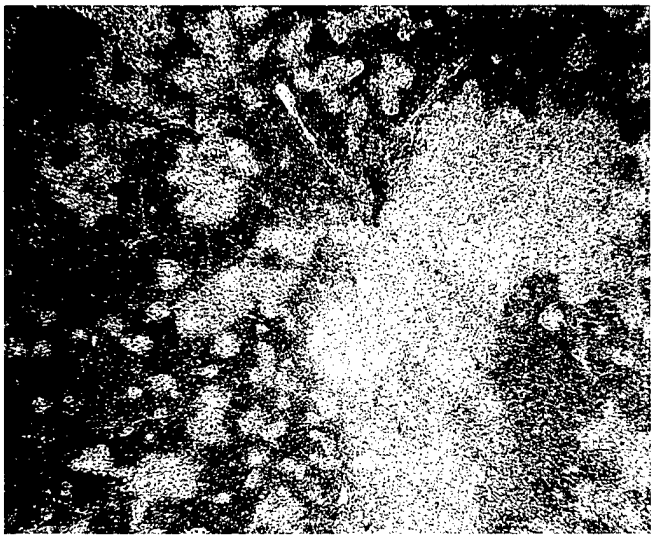

Figure 4. Epithelioid foci with associated necrosis and fibrosis in the bronchial lymph node 17 wk after end of exposure.

At 27 wk after end of exposure, the granulomas in the lung were more numerous and larger (Figure 5). At this time, the reaction could be considered a granulomatous pneumonia. The reaction in the bronchial lymph node continued to be severe.

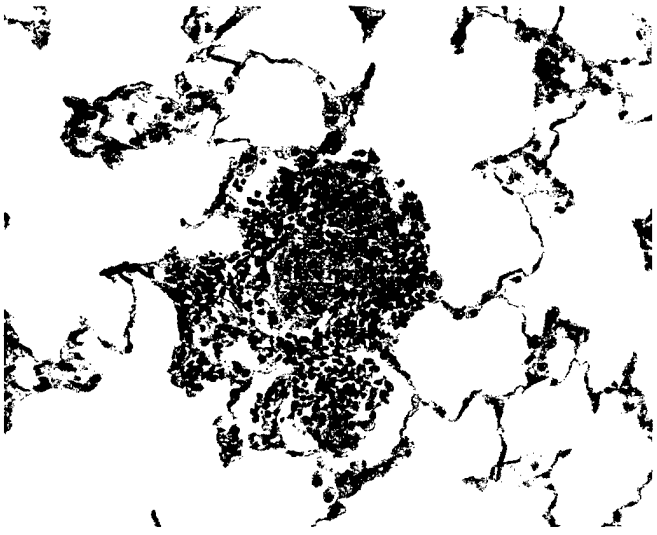

Figure 5. Granulomas in the lung parenchyma, characterized by epithelioid macrophages and moderate numbers of lymphocytes 27 wk after end of exposure.

At 37 wk after end of exposure, the number of granulomas had not increased, but the size and reactivity had decreased. There were fewer neutrophils and lymphocytes associated with the granulomas (Figure 6). Silica fibers could be found, but only with difficulty.

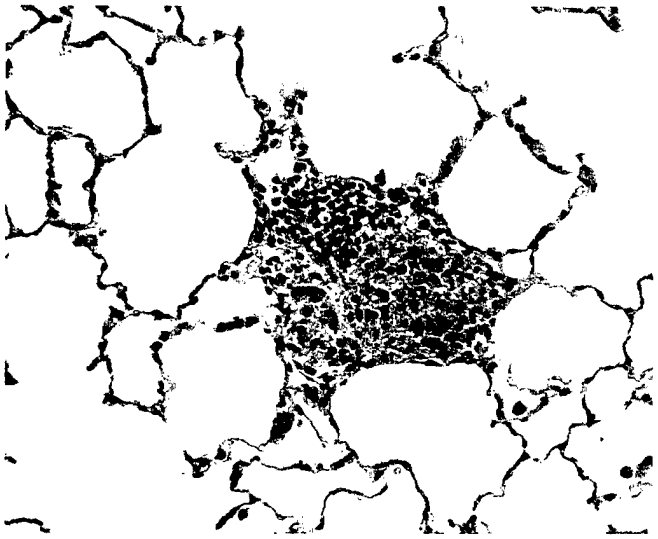

Figure 6. Granulomas in the lung parenchyma, characterized by epithelioid macrophages and a few lymphocytes 37 wk after end of exposure.

The complete listing of the severity grades for all rats is noted in Table 2.

Table 2
Microscopic Abnormalities and Severity Grading

		Lung									BALT			BLN			
Rat #	SAC Date	Focal AM	Septal Lymphoid Agg.	Alveolar PMN/LYM	Granuloma	AEH	PV Lymph. Infilt.	Focal Chronic Hem.	Septal Fib.	Fibers	Present	Epithelioid Cells	Fibers	Fibers	Lymphoid Hyperp.	Epithelioid Cells	Present
B001	5/1/01	–	–	–	–	–	–	–	–	++	++	–	–	NA	NA	NA	No
B002	4 days	+	–	–	–	–	–	+++	–	+	++	–	–	–	–	–	Y
B003		+	–	–	–	–	+	+	–	+	+	–	–	NA	NA	NA	No
B004		–	–	–	–	–	–	–	–	+	+	–	–	NA	NA	NA	No
B005		–	–	–	–	–	–	–	–	+	No	NA	NA	–	–	–	Y
B006	5/29/01	–	+	–	–	–	–	+	–	+	++	–	–	NA	NA	NA	No
B007	4 wk	+	+	–	–	–	–	–	–	–	+	–	–	–	–	–	Y
B008		+	–	–	–	–	–	–	–	+	++	–	–	–	–	–	Y
B009		+	+	–	–	–	–	–	–	+	No	NA	NA	+	–	–	Y
B010		+	–	–	–	–	–	–	–	+	++	–	–	+	–	–	Y
B016	7/10/01	++	+	++	–	+	–	–	–	–	+++	++	+	Not Sampled			
B017	10 wk	++	–	++	–	+	–	–	+	++	No	NA	NA	Not Sampled			
B018		+	+	++	–	–	–	–	–	–	+	+	++	Not Sampled			
B019		+	+	+	–	+	–	–	–	+	+++	+	+	Not Sampled			
B020		+	+	+	–	–	–	–	–	–	No	NA	NA	Not Sampled			
B026	8/30/01	+++	+	++	–	–	–	–	–	+	+	–	–	NA	NA	NA	No
B027	17 wk	+++	+	+++	+	+	–	–	+	+	+	+	–	++	++	++++	Y
B028		+++	+	+++	+	+	–	–	+	++	++	++	–	++	+++	++++	Y
B029		+++	+	+++	–	+	–	–	+	–	++	–	–	++	++	++++	Y
B030		+++	++	+++	–	+	–	–	+	+	+	+	–	++	++++	++++	Y photo
B031	11/9/01	+++	++	+++	++	+	+	–	+	+	++	+	+	++	++++	++++	Y
B032	27 wk	+++	+	+++	+++	+	+	–	+	+	++	+	+	++	+++	++++	Y
B033	Missing													+	+	++++	Y
B034		++	–	++	+	+	+	–	–	+	+	+	+	++	+++	++++	Y
B035		++	+	++	++	+	–	–	+	+	+	++	+	++	++	++++	Y
C021	1/15/02	+	+	+	++	++	+	–	+	+	+	+	+	Not Sampled			
C022	37 wk	++	+	+	++	++	–	–	+	–	+	++	–	Not Sampled			
C025		++	+	+	++	+	+	–	+	–	–	–	–	Not Sampled			
C026		+	+	+	++	+	–	–	+	–	–	–	–	Not Sampled			
Controls																	
D011	5/1/01	–	–	–	–	–	–	–	–	–	+	–	–	NA	NA	NA	No
D012	4 days	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	Y
D013		–	–	–	–	–	–	–	–	–	+	–	–	NA	NA	NA	No
D014		–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	Y
D015		–	–	–	–	–	–	–	–	–	No	NA	NA	NA	NA	NA	No
D041	11/9/01	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	Y
D042	27 wk	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	Y
D043		–	–	–	–	–	–	–	–	–	No	NA	NA	Not Sampled			
D044		+	–	–	–	–	–	–	–	–	No	NA	NA	–	–	–	Y
D045		–	–	–	–	–	–	–	–	–	+	–	–	NA	NA	NA	No
A041	1/15/02	–	–	–	–	–	–	–	–	–	+	–	–	Not Sampled			
A042	37 wk	–	–	–	–	–	+	–	–	–	No	NA	NA	Not Sampled			
A043		+	–	–	–	–	–	–	–	–	+	–	–	Not Sampled			
A044		–	–	–	–	–	–	–	–	–	+	–	–	Not Sampled			